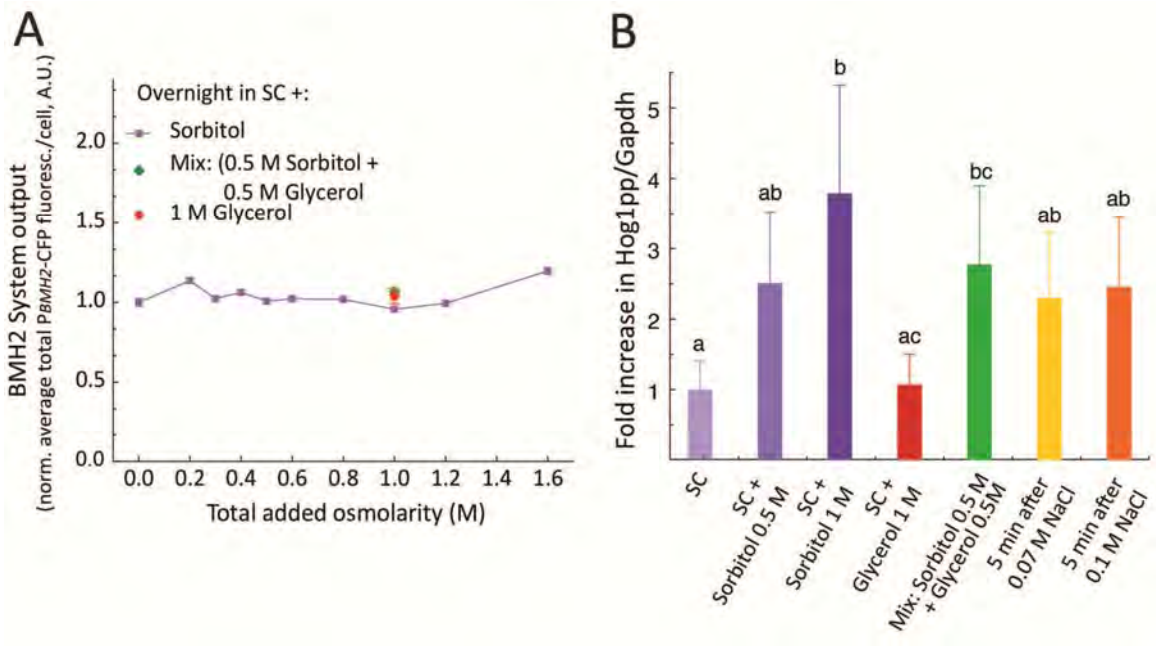

Figure S1. Behavior of the constitutive control reporter in cells adapted to high external osmolarity and comparison of Hog1 phosphorylation in cells acutely shocked and cells adapted to high osmotic strength media.

Data are from the same cells as in Figure 2A. A. Wild-type (LD3342) cells were grown in SC medium supplemented with: (i) increasing concentrations of sorbitol, (ii) SC + 1 M glycerol or (iii) SC + (0.5 M glycerol + 0.5 M sorbitol). Data corresponds to fold increase over the value measured in SC medium of the average fluorescence intensity of the P_{BMH2}-CFP reporter per cell. There is no significant increase of CFP with osmolarity ($p = 0.48$). Error bars represent the standard error of the mean (n ~ 700 cells for each data point). B. Steady state Hog1-pp for different added osmoticum. Same WB as in Figure 2B. Shared letters between bars indicate non-significant differences (α = 0.05 Tukey's multiple comparison test).

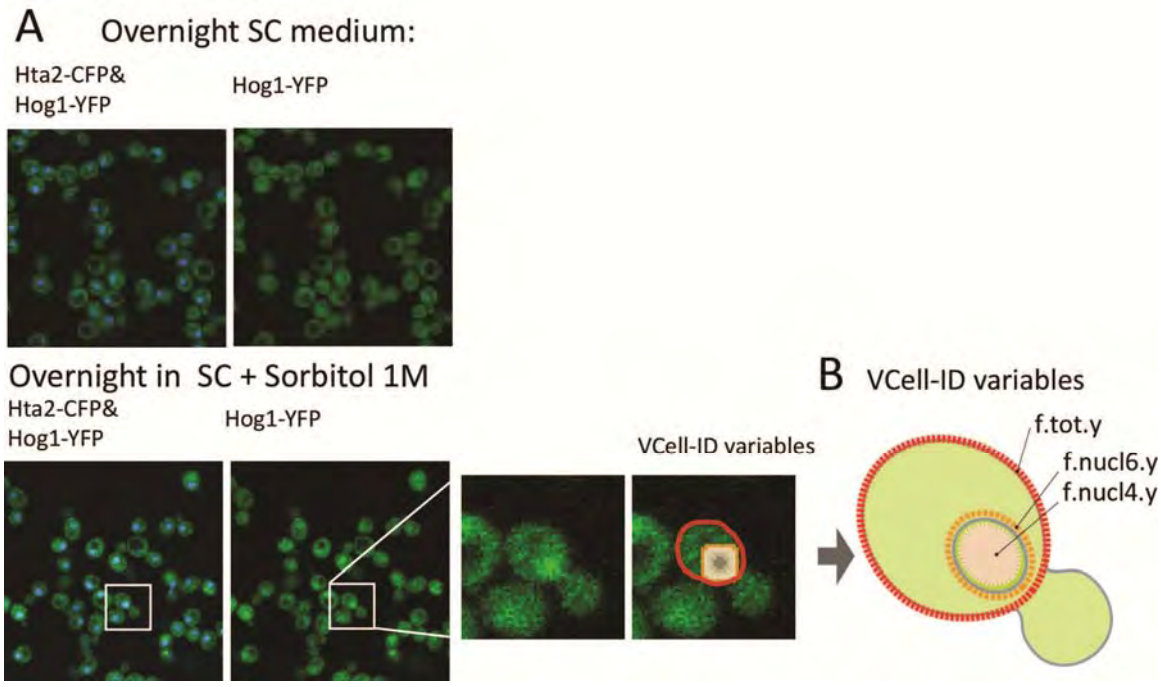

Figure S2. Quantification criteria for Hog1 subcellular localization.
Wild-type (ySP69) cells were grown in SC plus the indicated concentrations of sorbitol for at least 16 hours to ensure complete adaptation to high osmolarity (final OD ≤ 0.1). Images of cells and data from the plot are from the same experiment as in Figure 2C and S3. A. Exponentially growing ySP69 cells (expressing Hta2-CFP and Hog1-Venus) were cultured in SC (top) or SC + 1 M sorbitol (bottom). Right images show YFP channel and left images show the corresponding merged image to highlight the location of the nucleus. Zoomed-in image shows one cell with a white rectangle on top of the nucleus, corresponding to the VCell-ID variables used in the plot shown in (B). B. VCell-ID variables used for quantification. Diagram shows variables *f.nucl4.y*, *f.nucl6.y* and *f.tot.y* of indifferent regions of the cell zoomed-in in (A). Variables f.nucl4 and 6 correspond to the sum of the intensity in a disc of radius 5, and 7 pixels centered in the region of maximum intensity in the CFP channel. *f.tot* corresponds to the fluorescence of all pixells inside the cell. *f.nucl6.y* includes the fluorescence inside the total area of the nucleus in all cells analyzed. Therefore, in Figure 2C we calculated the cytoplasmatic fluorescence intensity as (*f.tot*.y/*a.tot*) - (*f.nucl6.y*/*a.nucl6*) and Hog1-Venus nuclear intensity as *f.nucl4*/*a.nucl4*.

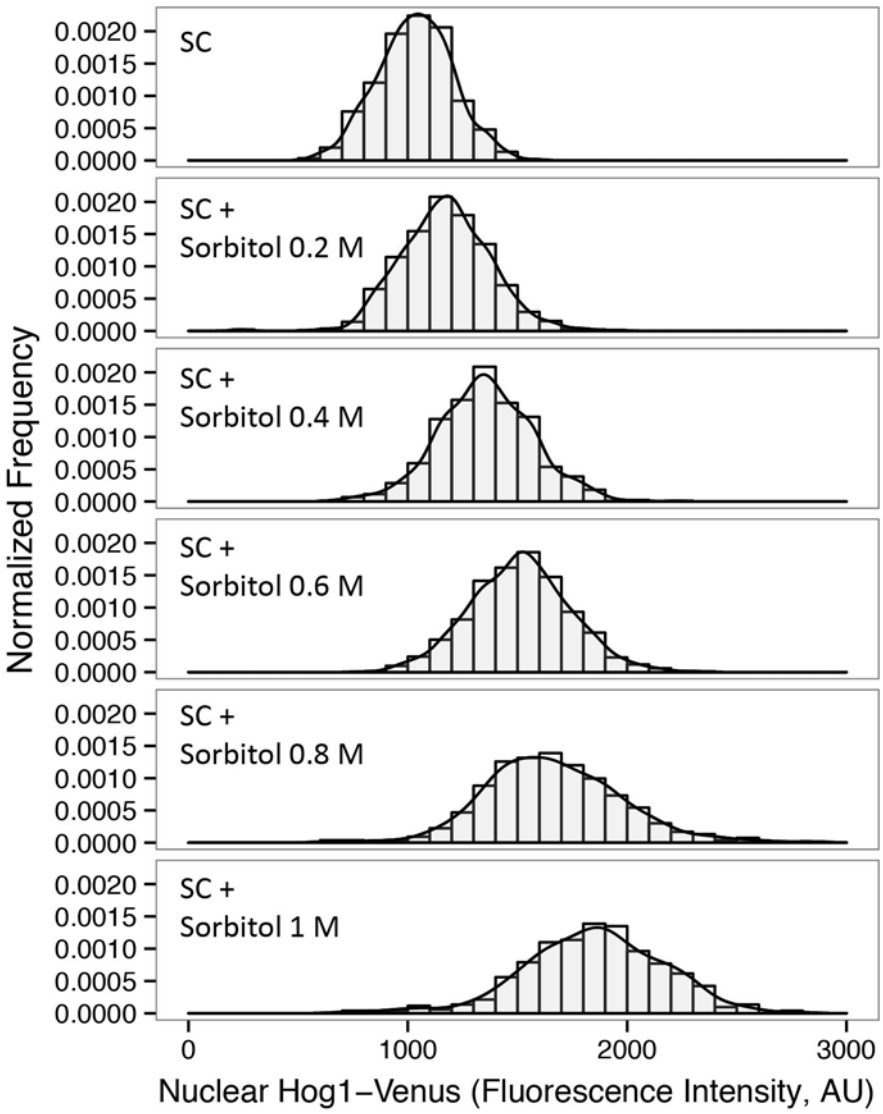

Figure S3. Hog1-Venus nuclear localization at different external osmolarities in single cells. Data corresponds to the same cells as Figure 2C. ySP69 cells (wild-type) expressing Hog1-Venus and Hta2-CFP (nucleus tag) as their sole source of Hog1 and Hta2 were grown overnight in SC medium supplemented with increasing concentrations of sorbitol. A to C. Histograms showing cell to cell variability in nuclear Hog1-venus in cells adapted to the indicated osmolarities. Plot shows data amalgamated from three biological replicates.

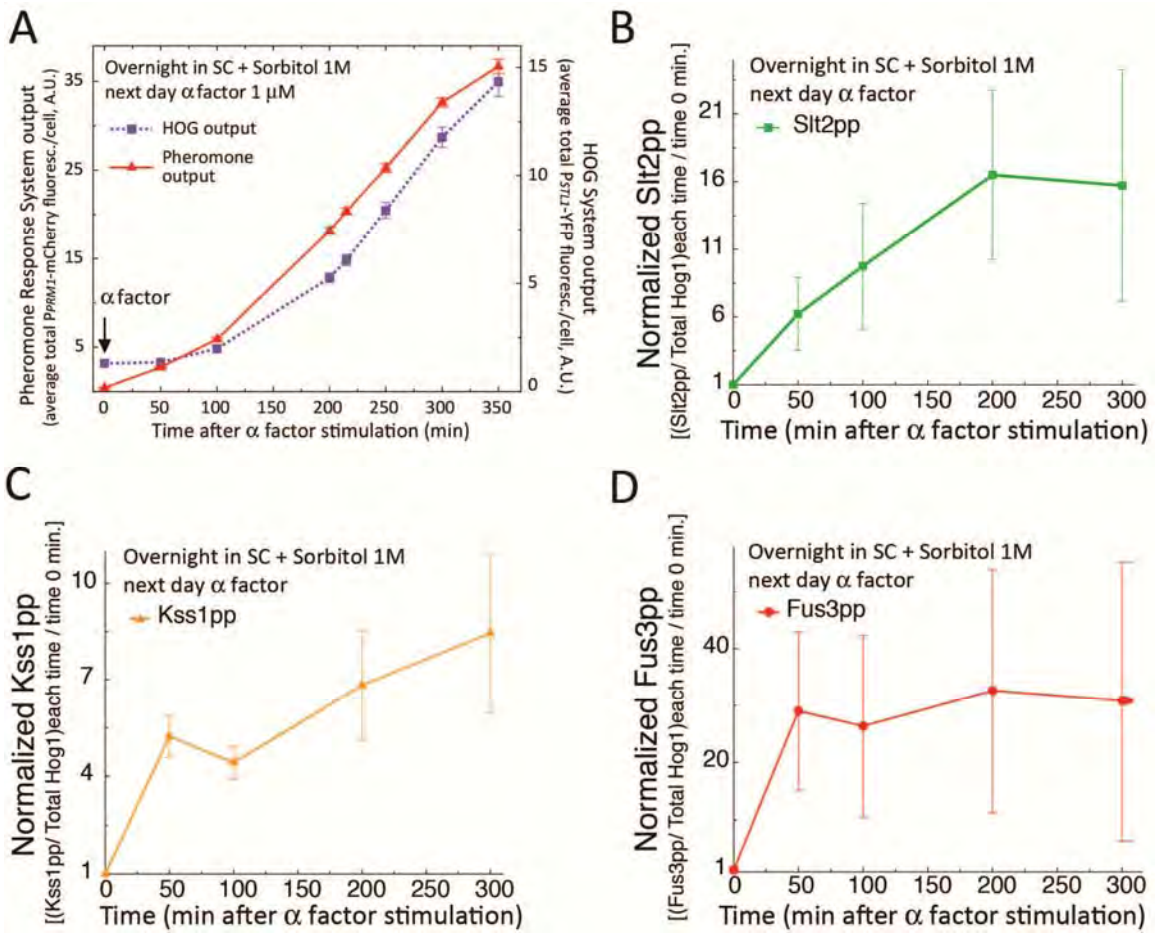

Figure S4. Pheromone response outputs during HOG activation induced by α factor.
Wild-type (LD3342) cells were grown in SC + 1 M sorbitol medium for at least 16 h to ensure complete adaptation to high osmolarity (A. OD ≤ 0.1, B-D. OD ≤ 0.25). On the following day cells were stimulated with 1 μM α factor. (A) HOG and PR transcriptional outputs. Data corresponds to the average ± SEM of YFP and mCherry fluorescence per cell, from a representative experiment. (N = 3). (B to D) Quantification of phosphorylation of Slt2, Kss1 or Fus3 as a function of time using data amalgamated from three biological replicates of the Western blots in Fig. 3.

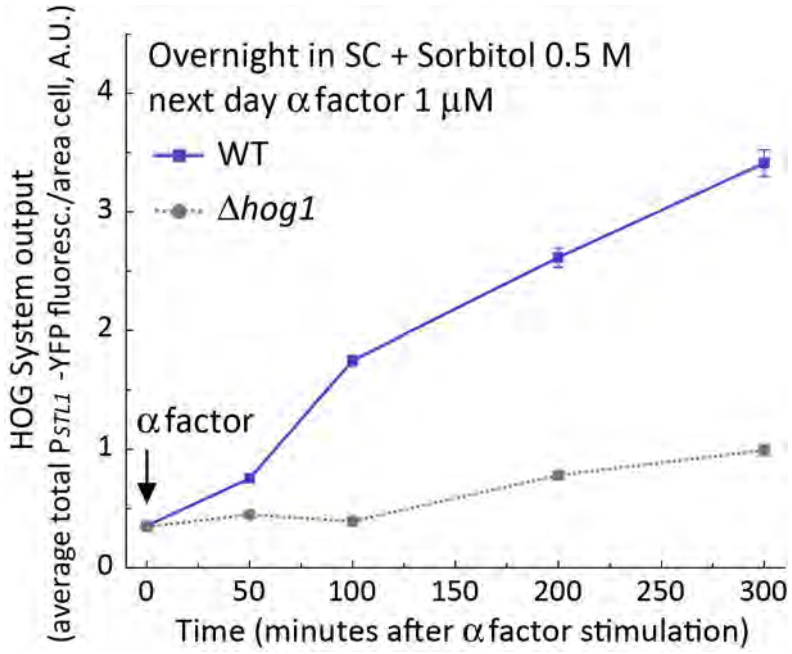

Figure S5. Δ *hog1* cells do not induce P_{STL1}-*YFP* in response to pheromone. Wild-type or Δ *hog1* (RB3801) cells were grown in SC medium supplemented with 0.5 M sorbitol for at least 16 hours. Several dilutions were started to obtain cells in exponential phase (OD ≤ 0.1) after overnight growth. The following day cells were stimulated with 1 μM α factor and samples were collected into cycloheximide for imaging. Data corresponds to average P_{STL1}-YFP fluorescence intensity per cell. Error bars represent the standard error of the mean. YFP expression rate is lower in Δ *hog1* as compared to WT cells ($p < e\text{-}4$). ($n > 1000$ cells). Plot shows data amalgamated from three biological replicates.

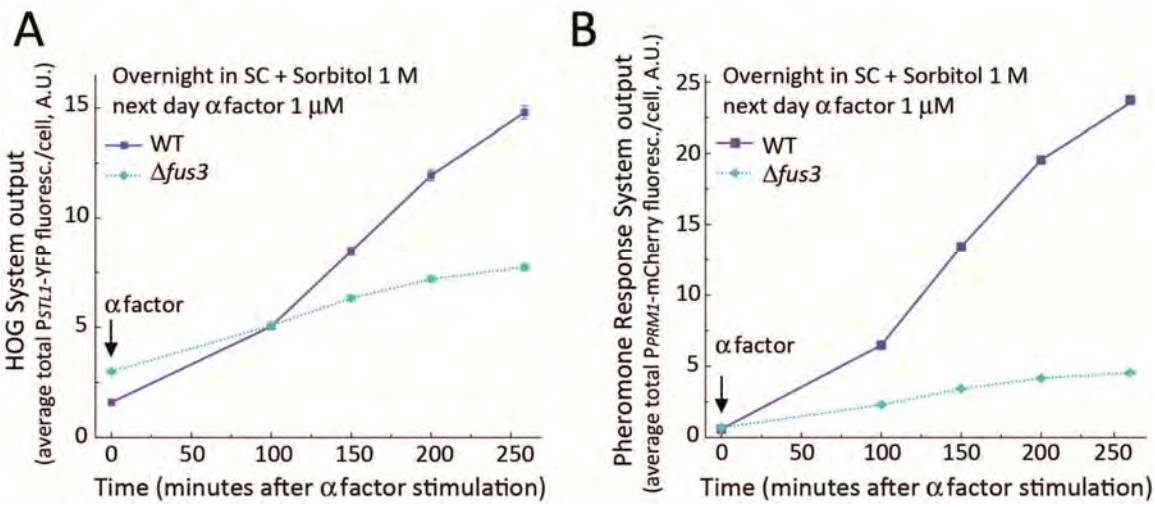

Figure S6. Fus3 is necessary for the pheromone-induced activation of HOG.
Wild-type (LD3342) or Δ*fus3* (RB3712) cells were grown in SC + 1 M sorbitol medium for at least 16 hours to ensure that cells were fully adapted to high osmolarity (OD ≤ 0.1). On the following day cells were stimulated with 1 μM α factor. (A) HOG transcriptional output in wild-type is higher than in Δ*fus3* cells ($p < e\text{-}4$). B. Pheromone response transcriptional output in wild-type is higher than in Δ*fus3* cells ($p < e\text{-}4$). Data corresponds to the average total YFP or mCherry fluorescence per cell. Error bars represent the standard error of the mean. (n ~ 700 cells). Plots show one representative experiment out of three biological replicates.

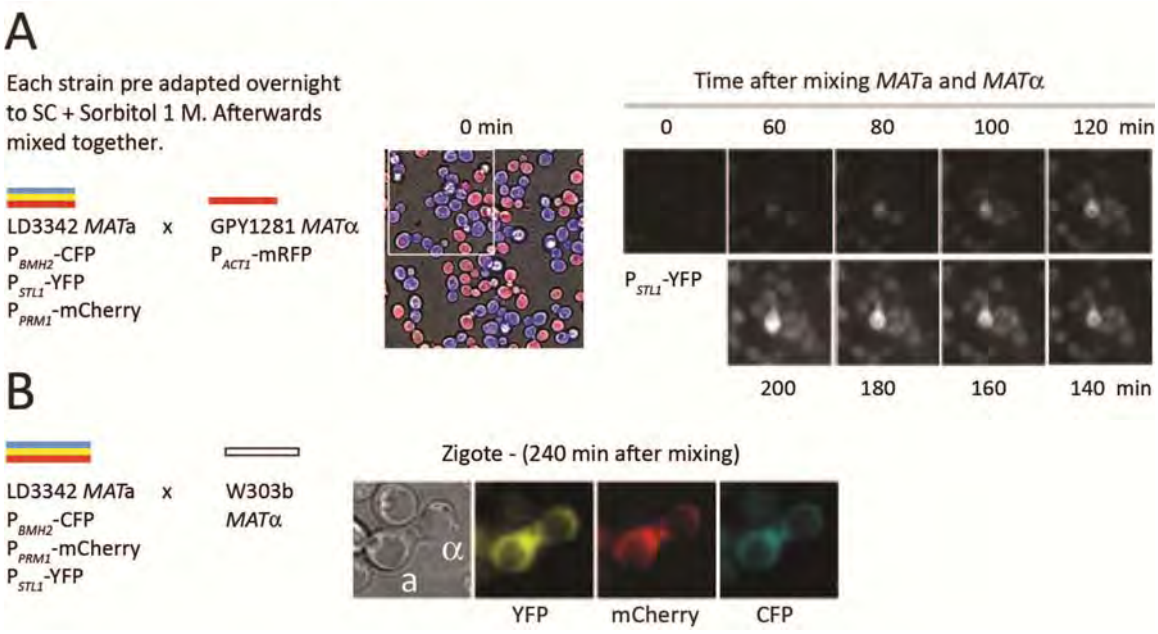

Figure S7. HOG transcriptional output is induced during mating in high external osmolarity.
Strains were adapted to SC media supplemented with 1 M sorbitol for at least 16 hours to ensure complete adaptation. A. Left: Scheme indicates the fluorescent reporters present in the strains used in the mating mix . Right: Images were taken at different times after mixing. The images show the expression of the HOG reporter (P_{STL1}-YFP) over time. Center: Brightfield image with the CFP and mCherry channels superimposed. Cyan cells are *MAT*a and red cells are *MAT* α (*MAT*a cells have not had time to induce the pheromone controlled P_{PRM1}-mCherry reporter). B. Left. Schema of the mating mix: Wild-type W303 *MAT*a (LD3342) x wild-type W303b *MAT* α . Right. After 240 minutes of mating, cells were imaged for zygotes. In the example shown, the cells express the P_{STL1}-YFP and P_{PRM1}-mCherry reporters (both in the *MAT*a cell) as well as the constitutive reporter P_{BMH2}-CFP**.**

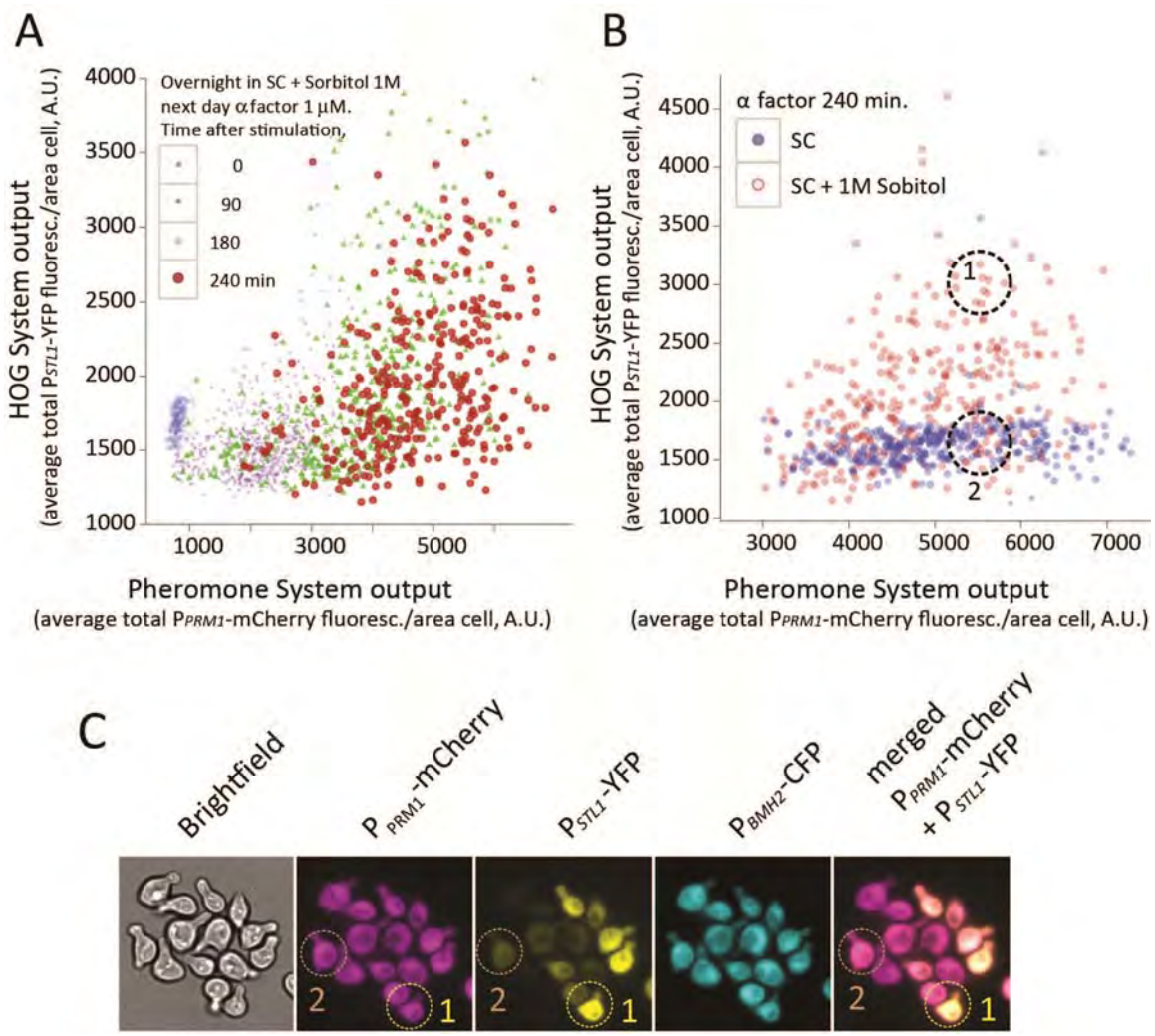

Figure S8. Low correlation between the PR and HOG reporters.

Wild type (LD3342) cells were grown in SC + 1 M sorbitol (A) or SC (B) medium for at least 16 hours to ensure complete adaptation to high osmolarity (OD ≤ 0.1). Subsequently cells were stimulated with 1 μ M α factor. A. Scatter plot showing HOG compared to PR transcriptional outputs. Each data point corresponds to the signal of an individual cell at a given time after stimulation with α factor. At 240 min all cells activated PR dependent transcription (all circles are on the high side of the x axis) but many cells do not show HOG activation (some circles are low and some are high in the y axis). B. Activation of HOG by pheromone requires high osmolarity. Same experiment as in (A), but including data obtained at 240 min from cells grown in SC medium without sorbitol or in SC + 1 M sorbitol. In SC medium there is no activation of the HOG reporter. In SC + 1 M sorbitol medium, activation of the HOG reporter showed large cell to cell variation. The correlation between P_{STL1}-YFP and P_{PRM1}-mCherry (ρ = 0.61 $\pm$ 0.05) was significantly lower than the correlation between P_{PRM1}-mCherry and P_{BMH2}-CFP (ρ = 0.92 $\pm$ 0.01), for all pheromone doses and times tested ($p < 0.001$). C. Montage showing images of cells with the transcriptional reporters expressed to various degrees. Images correspond to the cells shown in (B). Circles highlight one cell from region 1 and one cell from region 2 in (B). The image was colored as follows: mCherry in purple, YFP in yellow, and CFP in cyan.

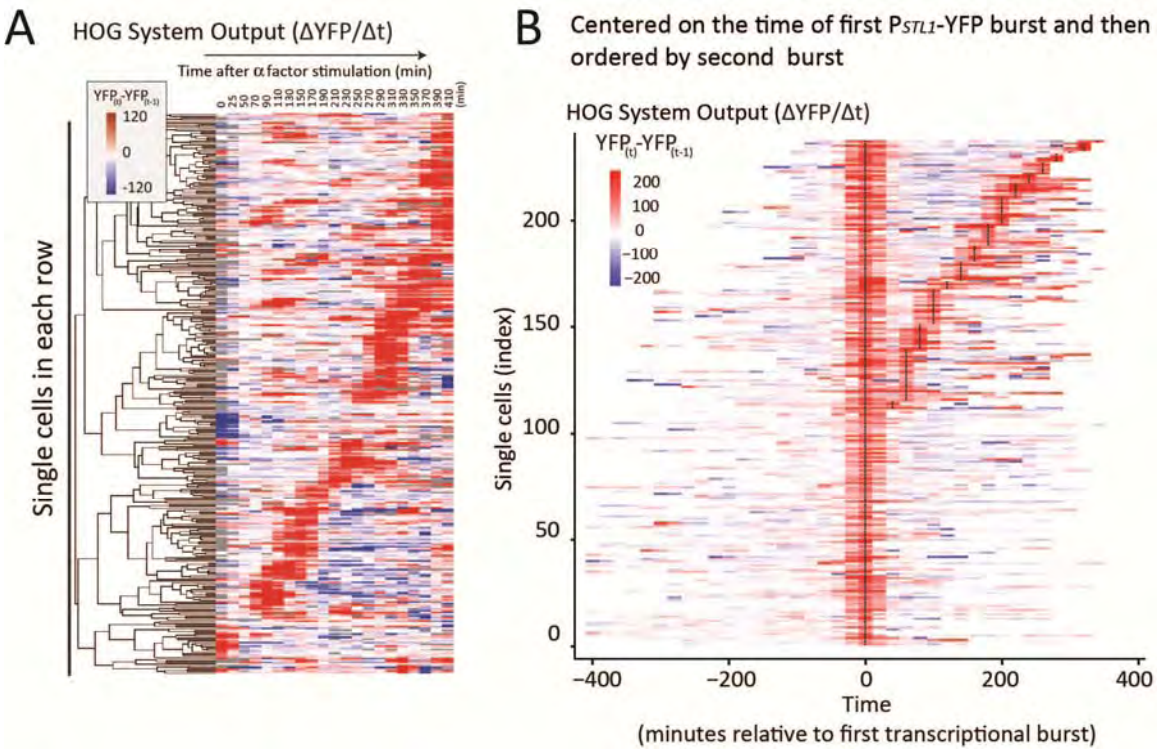

Figure S9. Alternative ordering of cells based on HOG transcriptional bursts and the timing of shmoo formation.

A. Same experiment as in Figure 4. Hierarchical clustering of single cell HOG transcriptional output profiles. Cells were clustered based on the pattern of expression of the HOG reporter (P_{STL1}-YFP) over time, using the Pearson correlation to assess distance, and average linkage to build the tree. For clustering, we used "ΔYFP/Δt", the time derivative of the YFP fluorescence density. Rows correspond to cells and columns to time interval. Data is displayed as a clustered heat-map of cells. B. Non-clustering-based ordering of the cells. Wild-type (LD3342) cells were grown overnight in SC plus 1 M sorbitol and then stimulated with 1 μM α factor and imaged over time as explained in Figure 4. Time course data for every cell was analyzed to find the time at which a HOG burst took place as explained in Figure S10. The detected times of burst are marked with a dot. We centered cells on time of their first burst and then sorted them (bottom-up) based on the time they show their second burst. Approximately half of the cells do not exhibit a clear second burst.

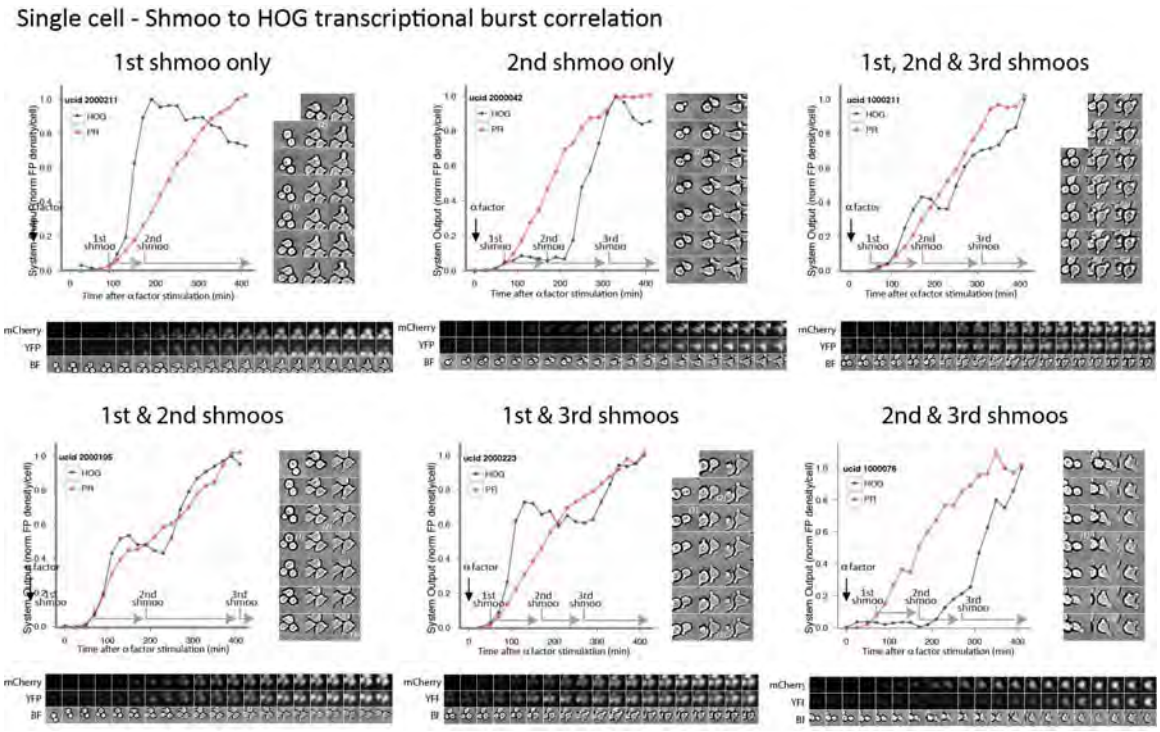

Figure S10. Correlation between shmooing and HOG output during pheromone-dependent HOG activation.

Additional examples from the experiment shown in Figure 4. Wild-type (LD3342) cells were grown overnight in SC plus 1 M sorbitol and then stimulated with 1 μM α factor and imaged over time as explained in the legend to Figure 4. Single cell transcriptional output after α factor stimulation as a function of time at high external osmolarity. Six representative cells highlighting the heterogeneity of responses are shown. Some cells activate HOG in the first shmoo, whereas others in the second or third, or in more than one shmoo. Plots show HOG and PR transcriptional output, overlaid with shmooing times (light purple L shaped arrows mark shmooing periods, starting at the time each mating projection is first apparent). Photomontage shows the brightfield time-lapse images used to determine the timing of shmooing. Numbers mark shmoos and arrows highlight the position of the nascent mating projection in each cell. Accumulation of the HOG reporter is slightly delayed (about 30 to 40 minutes) from the time of shmooing due to the slow maturation of YFP, which is about 40 minutes (*28*). Data corresponds to the fluorescence intensity normalized to the maximum and minimum values for each cell. Below each plot time lapse images obtained in the YFP channel (HOG reporter) are shown.

Correlation between HOG bursts and cell cycle

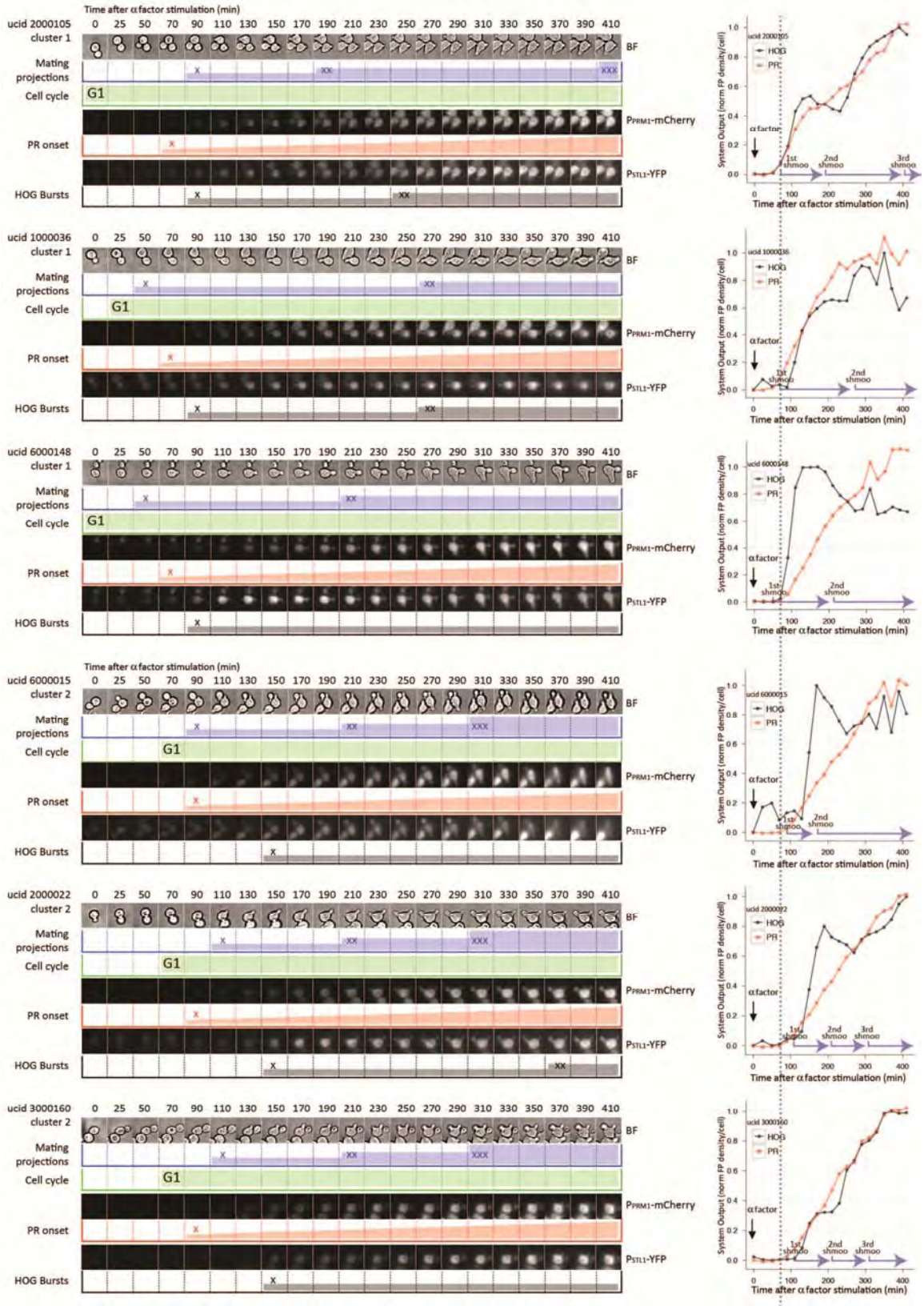

Figure S11. Effect of the cell cycle position on the timing of HOG activation by pheromone.
Same experiment as in Figures 4 and 5A to B. Wild-type (LD3342) cells were grown overnight in SC plus 1 M sorbitol, then stimulated with 1 μ M α factor and imaged over time. Six individual cells are shown: Three that activated HOG early in the time course (top) and three that activated it later (bottom). Left. Brightfield (top), PR output (center) and HOG output (bottom) images (*ucid* corresponds to the unique identifier given by our image analysis software VCell-ID). Blue: beginning of shmooing. Green: first frame in which a given cell is clearly in the G1 phase of the cell cycle. Red: Pheromone transcriptional output, highlighting the time frame of its onset (cross) and the increasing accumulation of mCherry over the time course. Gray: HOG transcriptional output highlighting transcriptional bursts (crosses). Right. Plots showing quantification of HOG and PR transcriptional outputs, overlaid with shmooing times (light purple L shaped arrows mark shmooing periods, starting at the time each mating projection is first apparent). A vertical dotted line was overlaid at the time of the first HOG burst on the top cell to help compare the timing of activation in different cells.

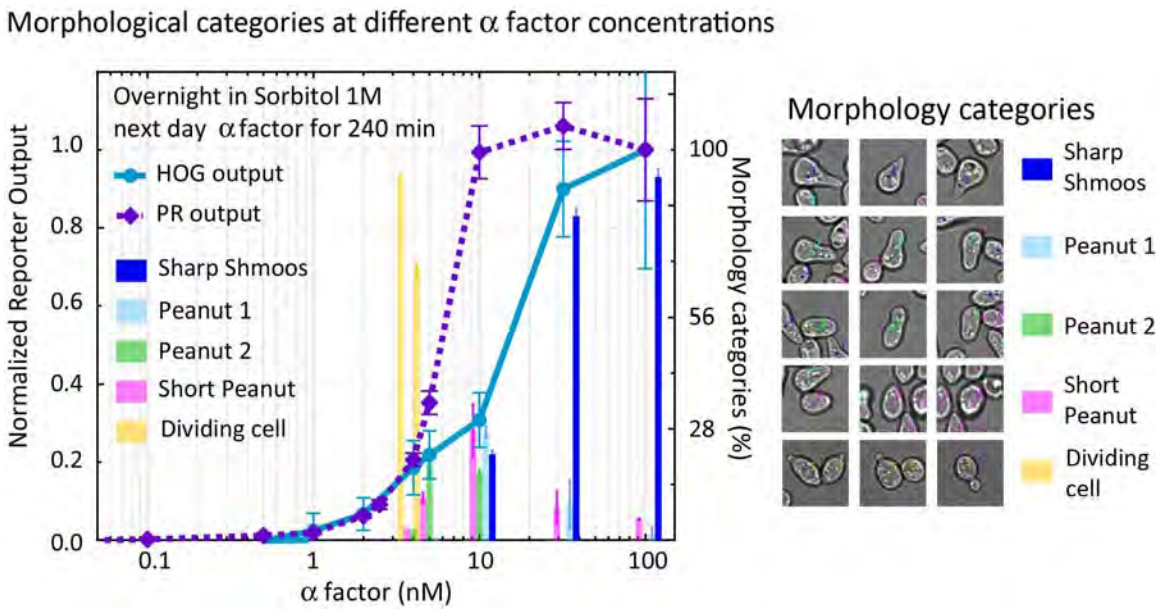

Figure S12. Mating projection classification and their distribution in response to different pheromone concentrations.

Same experiment as in Figure 6B. Wild-type (LD3342) cells were grown in SC + 1 M sorbitol medium for at least 16 hours to ensure complete adaptation to high osmolarity (OD ≤ 0.1). On the following day cells were treated with the indicated pheromone concentrations. After 4 hours, samples were collected for imaging in cycloheximide. Left. Data corresponds to the average YFP (circles) or mCherry (diamonds) normalized to maximum value (left Y axis); or the percentage of cells with the different mating projection morphology categories 1 to 5 (right Y axis). Right. The image corresponds to cells after 180 minutes of α factor stimulation showing representative cells of each category. Cells showing a sharp mating projection with concave curvature at the projection base were considered sharp shmoos, cells with an elongated shape were considered peanuts. We considered cells with asymmetric shape and cell diameter narrowing in the middle as peanut I, and cells more symmetric and without a narrowing in the middle were classified as peanut II. The less elongated cells were classified as growing peanuts. Budding cells were classified as dividing cells. We quantified two fields for every pheromone concentration. Error bars represent standard error of the mean for transcription and SD for morphology. Plots show a representative experiment out of three biological replicates.

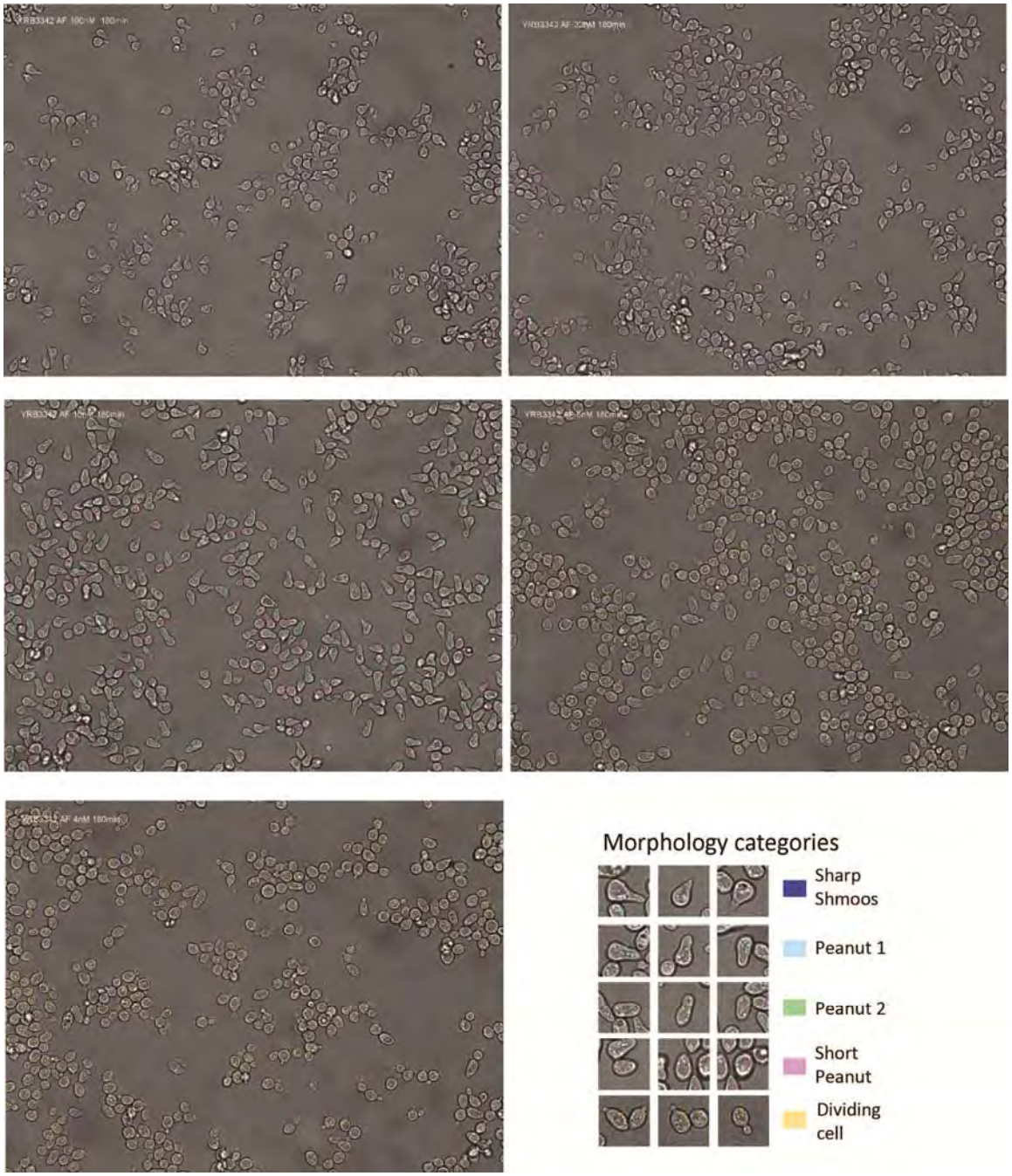

Figure S13. Examples of mating projection classification at different pheromone concentrations.
Data from the experiment in Figure 6B. Wild-type (LD3342) were grown in SC + 1M sorbitol medium for at least 16 hours to ensure complete adaptation to high osmolarity (OD < 0.1). On the following day cells were treated with the indicated pheromone concentrations. Images correspond to 5 fields treated with pheromone concentrations : 4, 5, 10, 30 and 100 nM for 180 minutes. Mating projection morphology categories. (1) Shmoo: cells showing a sharp mating projection with concave curvature at the projection base were considered sharp shmoos. (2) Peanut I: elongated cells with asymmetric shape and cell width narrowing in the middle. (3) Peanut II: elongated cells that were more symmetric and without a narrowing in the middle. (4) Growing peanut: less elongated cells. (5) Budding cells were classified as dividing cells.

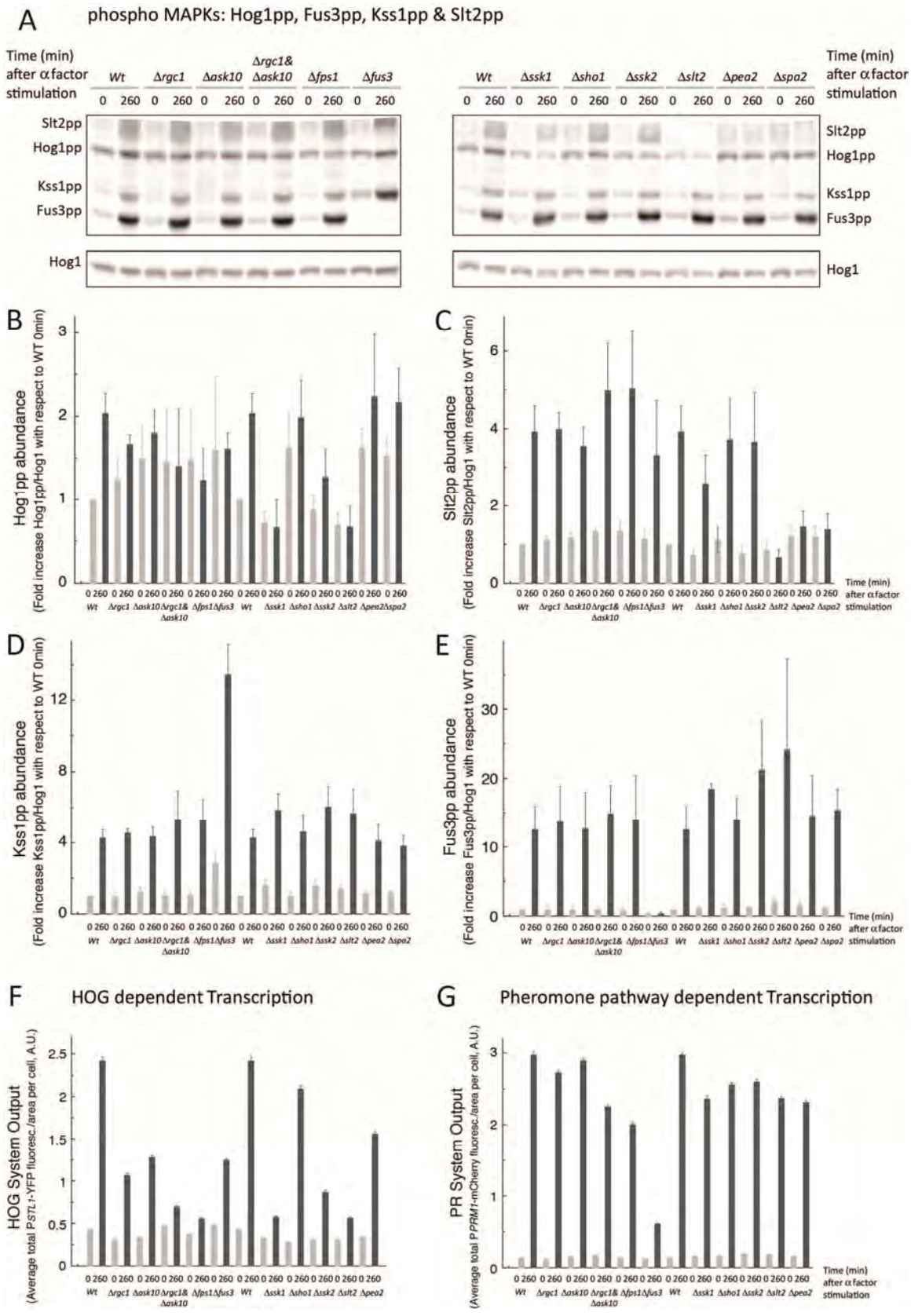
A
phospho MAPKs: Hog1pp, Fus3pp, Kss1pp & Slt2pp
Time (min) after α factor stimulation
Wt
Δrgc1
Δask10
Δrgc1& Δask10
Δfps1
Δfus3
Δssk1
Δsho1
Δssk2
Δslt2
Δpea2
Δspa2
0 260
Slt2pp
Hog1pp
Kss1pp
Fus3pp
Hog1
B
Hog1pp abundance
(Fold increase Hog1pp/Hog1 with respect to WT 0min)
C
Slt2pp abundance
(Fold increase Slt2pp/Hog1 with respect to WT 0min)
D
Kss1pp abundance
(Fold increase Kss1pp/Hog1 with respect to WT 0min)
E
Fus3pp abundance
(Fold increase Fus3pp/Hog1 with respect to WT 0min)
F
HOG dependent Transcription
HOG System Output
(Average total PSTL1-YFP fluoresc./area per cell, A.U.)
G
Pheromone pathway dependent Transcription
PR System Output
(Average total PPRM1-mCherry fluoresc./area per cell, A.U.)

Figure S14. Quantification of MAPKs phosphorylation in WT and mutant strains in response to pheromone.

Wild-type (LD3342) or mutant cells were grown in SC + 1 M sorbitol medium (or 1 M glycerol) for at least 16 hours to assure complete adaptation to high osmolarity (OD ≤ 0.2). On the following day cells were stimulated with 1 μM α factor. A. Western blot against phosphorylated Hog1, Fus3, Kss1, Slt2 and total Hog1. B. HOG phosphorylation quantification. Hog1 dual phosphorylation at times 0 and 260 min after α factor stimulation in all mutants. C to E. Pheromone response and CWI phosphorylation outputs. Dual phosphorylation of Fus3, Kss1 and Slt2 at times 0 and 260 min after α factor stimulation in all mutants. F. HOG transcriptional output. Average P_{STL1}-YFP fluorescence per cell. Error bars represent standard error of the mean. G. Pheromone response transcriptional output. Average P_{PRM1}-mCherry fluorescence per cell. Error bars represent standard error of the mean. Strains used: Wild type (LD3342), Δ*sho1* (RB3704), Δ*ssk1* (RB3382a), Δ*ssk2* (RB3642) and Δ*fus3* (RB3713), Δ*fps1* (RB3396a), Δ*rgc1* (RB3710), Δ*ask10* (RB3717) and Δ*rgc1* Δ*ask10* (RB3722), Δ*pea2* (RB3865), Δ*spa2* (RB3862) and Δ *slt2* (RB3376a). A to G. Samples from the same experiments. Data amalgamated from three biological replicates.

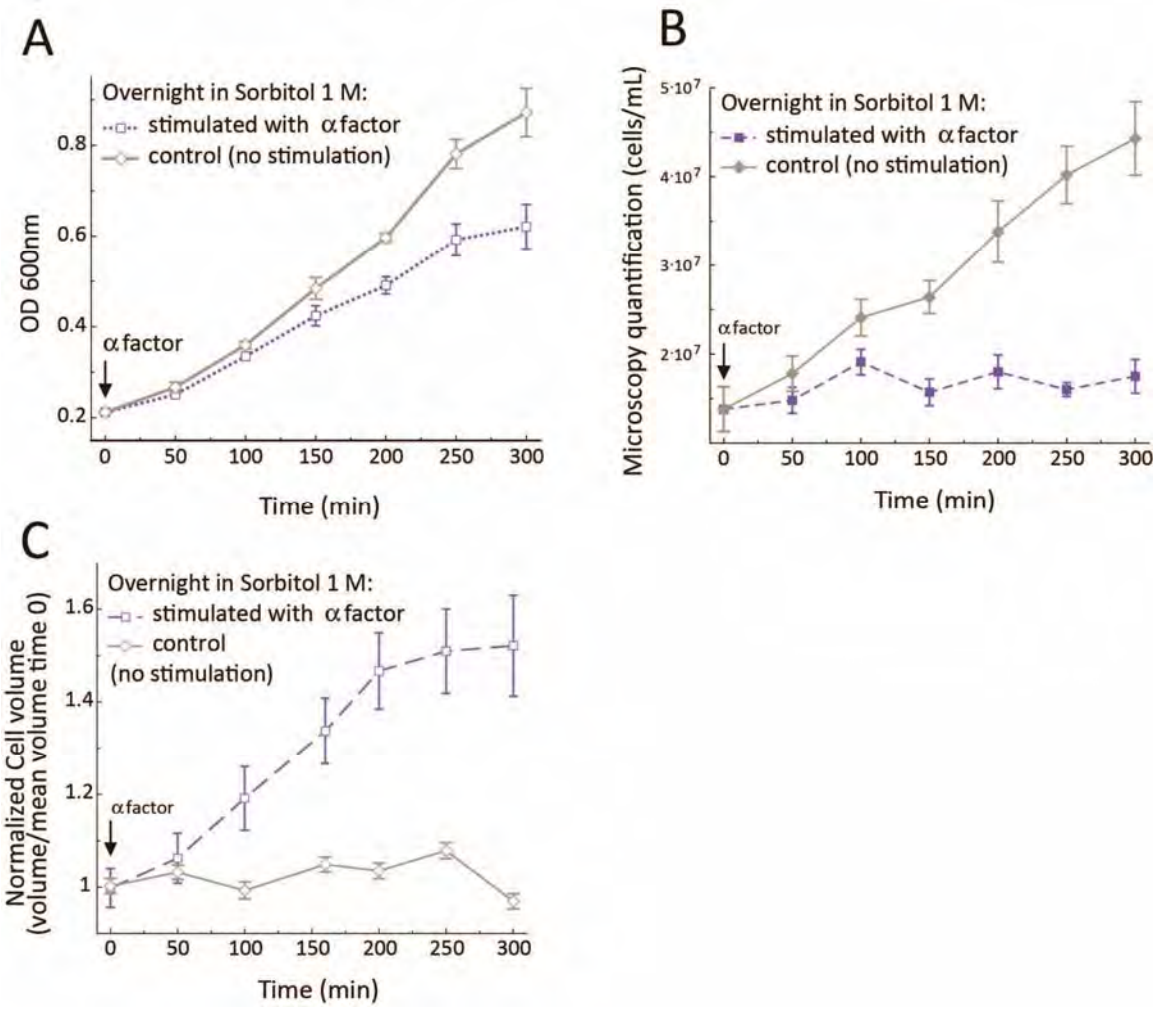

Figure S15. Additional data from the extracellular glycerol accumulation experiments. Data is from the experiments in Figure 7B. Wild-type cells were grown in SC + 1 M sorbitol medium for at least 16 hours to ensure complete adaptation to high osmolarity (OD ≤ 0.2). Plots show, A. optical density (OD 600nm), B. number of cells and C. average of cell volume normalized to the average volume at time 0 of the cultures treated (squares) or not (diamonds) with 1 μ M pheromone as a function of time. Error bars represent SD of the average of three independent experiments. All the experiments were started at the same optical density.

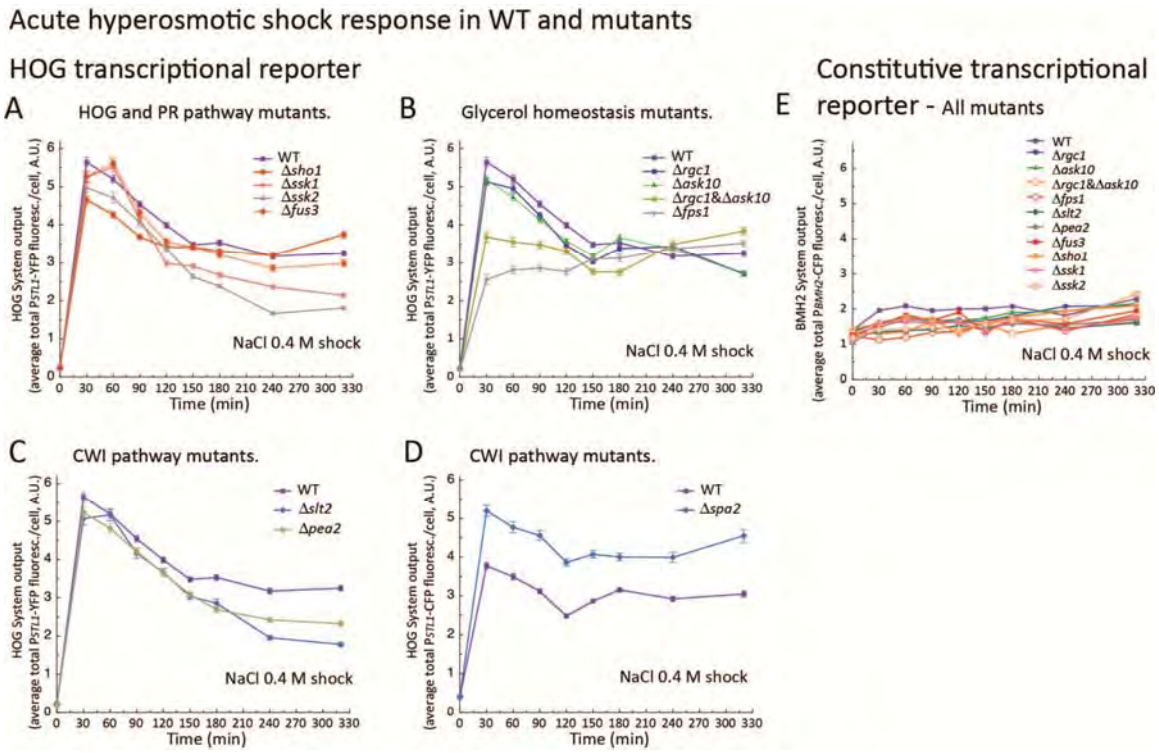

Figure S16. HOG reporter activation in WT and mutant strains in response to an acute hyperosmotic shock.

Cells were grown in SC medium for at least 16 hours. Several dilutions were started to obtain cells in exponential phase (OD ≤ 0.1) after overnight growth before exposing suitable cultures to an acute 0.4 M NaCl hyperosmotic shock. A to D. HOG transcriptional output. A. HOG pathway deletion mutants and Δ*fus3*. Strains: Wild-type (LD3342), Δ*sho1* (RB3704), Δ*ssk1* (RB3382a), Δ*ssk2* (RB3642) and Δ*fus3* (RB3713). (B) Glycerol homeostasis deletion mutants. Strains: Wild-type (LD3342), Δ*fps1* (RB3396a), Δ*rgc1* (RB3710), Δ*ask10* (RB3717) and Δ*rgc1* Δ*ask10* (RB3722). C and D. CWI pathway mutants. Strains: Wild-type (LD3342 or RB3406), Δ*pea2* (RB3865), Δ*spa2* (RB3862) and Δ*slt2* (RB3376a). E. BMH2 transcriptional output measured in the same cells of experiments a to d as control. Points in A to E represent average fluorescence of each transcriptional reporter per cell. Error bars represent standard error of the mean. Plots show a representative experiment out of three biological replicates.

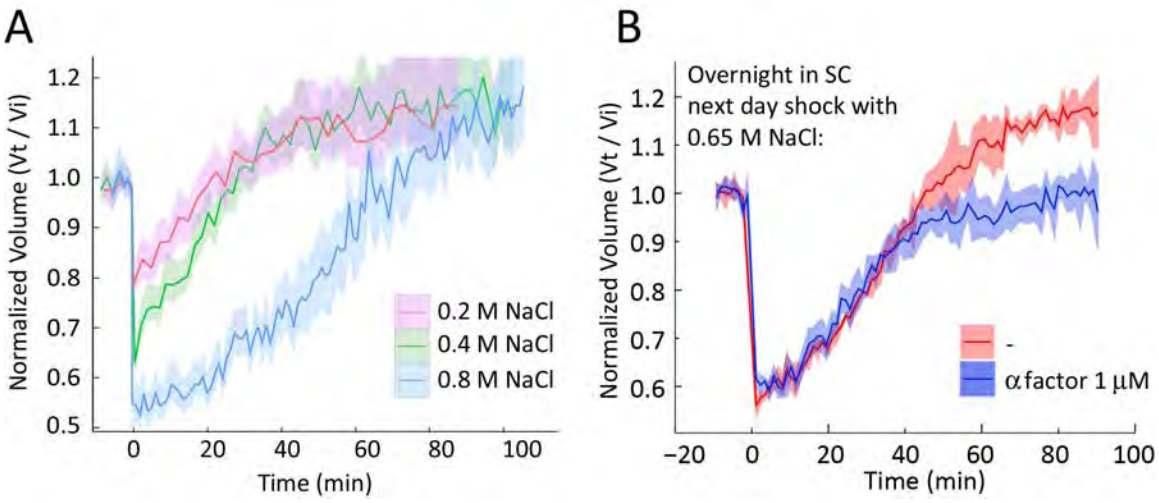

Figure S17. Additional volume recovery experiments.

Wild-type (LD3342) cells were grown in SC medium for at least 16 hours (cells were in exponential phase OD ≤ 0.1), exposed to the indicated hyperosmotic shock and imaged over time to measure volume as detailed in supplementary methods. Data corresponds to the average normalized volume. Error bars represent standard error of the mean. Plots show a representative experiment of three biological replicates. A. Acute hyperosmotic shocks. 0.2 M NaCl, 0.4 M NaCl and 0.8 M NaCl. B. Acute hyperosmotic shock in cells growing in SC medium with or without α factor pretreatment. Cells pretreated or not with 1 μM α factor for 4 h and then shocked with 0.65 M NaCl. In contrast to the results in Fig. 8D, cells growing in SC and not adapted to high osmolarity conditions recover only slightly faster if treated with pheromone (0.88 ± 0.07 of the non-treated cells recovery time, p = 0.027). (N = 3).

Table S1. Statistical analysis of phosphorylation of MAPKs related to Fig. S14.

	Hog1pp/Hog1 Time=0			Hog1pp/Hog1 Time=260 min			Hog1pp/Hog1 absolute increase in 260 min			Hog1pp/Hog1 fold increase in 260 min		
Strain	**Mean**	**SE**	**p-value vs WT**	**Mean**	**SE**	**p-value vs WT**	**Mean increase**	**SE**	**p-value vs zero increase**	**fold increase**	**SE**	**p-value vs WT**
WT	0.108	7.6E-03	NA	0.218	0.008	NA	0.110	0.009	6.7E-16	2.039	0.091	NA
Δ*rgc1*	0.129	1.9E-02	8.8E-02	0.189	0.019	1.7E-02	0.061	0.013	3.3E-05	1.518	0.217	4.3E-04
Δ*ask10*	0.150	1.9E-02	8.7E-04	0.195	0.019	5.4E-02	0.045	0.013	1.4E-03	1.348	0.217	1.6E-05
Δ*rgc1*Δ*ask10*	0.130	1.9E-02	7.2E-02	0.128	0.019	6.5E-10	-0.002	0.013	8.8E-01	1.035	0.217	6.2E-08
Δ*fps1*	0.140	1.9E-02	8.6E-03	0.120	0.019	5.9E-11	-0.021	0.013	1.2E-01	0.906	0.217	7.9E-09
Δ*fus3*	0.138	1.9E-02	1.6E-02	0.174	0.019	4.3E-04	0.036	0.013	9.0E-03	1.342	0.217	1.5E-05
Δ*ssk1*	0.074	1.9E-02	6.3E-03	0.069	0.019	4.8E-17	-0.006	0.013	6.8E-01	0.862	0.217	4.0E-09
Δ*sho1*	0.164	1.9E-02	1.9E-05	0.202	0.019	1.8E-01	0.038	0.013	6.0E-03	1.191	0.217	9.1E-07
Δ*ssk2*	0.090	1.9E-02	1.4E-01	0.129	0.019	1.0E-09	0.039	0.013	4.7E-03	1.388	0.217	3.5E-05
Δ*slt2*	0.071	1.9E-02	3.0E-03	0.069	0.019	4.7E-17	-0.002	0.013	8.5E-01	0.933	0.217	1.2E-08
Δ*pea2*	0.166	1.9E-02	1.1E-05	0.225	0.019	5.5E-01	0.059	0.013	4.4E-05	1.304	0.217	7.2E-06
Δ*spa2*	0.155	1.9E-02	2.2E-04	0.220	0.019	8.6E-01	0.065	0.013	1.0E-05	1.372	0.217	2.5E-05
	Slt2pp/Hog1 Time=0			**Slt2pp/Hog1 Time=260 min**			**Slt2pp/Hog1 absolute increase in 260 min**			**Slt2pp/Hog1 fold increase in 260 min**		
Strain	**Mean**	**SE**	**p-value vs WT**	**Mean**	**SE**	**p-value vs WT**	**Mean increase**	**SE**	**p-value vs zero increase**	**fold increase**	**SE**	**p-value vs WT**
WT	0.612	3.2E-02	NA	2.368	0.095	NA	1.756	0.099	6.1E-23	3.939	0.174	NA
Δ*rgc1*	0.649	8.7E-02	5.1E-01	2.313	0.256	7.3E-01	1.664	0.138	3.2E-16	3.549	0.453	1.8E-01
Δ*ask10*	0.672	8.8E-02	2.9E-01	2.004	0.244	1.9E-02	1.332	0.125	2.4E-14	2.972	0.442	1.6E-03
Δ*rgc1*Δ*ask10*	0.732	9.1E-02	4.9E-02	2.603	0.267	1.8E-01	1.872	0.153	1.6E-16	3.567	0.453	2.0E-01
Δ*fps1*	0.739	9.1E-02	3.9E-02	2.662	0.269	9.9E-02	1.923	0.155	1.1E-16	3.585	0.454	2.2E-01
Δ*fus3*	0.626	8.6E-02	7.9E-01	1.480	0.225	1.4E-08	0.854	0.100	3.3E-11	2.351	0.428	2.8E-06
Δ*ssk1*	0.436	7.7E-02	3.2E-04	1.542	0.227	1.1E-07	1.106	0.099	4.4E-15	3.549	0.452	1.8E-01
Δ*sho1*	0.663	8.8E-02	3.6E-01	2.227	0.253	3.8E-01	1.564	0.135	1.2E-15	3.382	0.449	5.5E-02
Δ*ssk2*	0.456	7.8E-02	1.5E-03	2.167	0.250	2.0E-01	1.711	0.128	6.6E-18	4.731	0.473	1.5E-02

Δ*slt2*	0.518	8.1E-02	6.4E-02	0.398	0.194	4.6E-25	-0.120	0.048	1.6E-02	0.831	0.386	7.7E-13
Δ*pea2*	0.735	9.1E-02	4.3E-02	0.873	0.206	4.4E-18	0.138	0.077	8.1E-02	1.209	0.398	3.0E-11
Δ*spa2*	0.725	9.1E-02	6.2E-02	0.828	0.204	8.7E-19	0.103	0.075	1.7E-01	1.179	0.397	2.3E-11
	Kss1pp/Hog1 Time=0			**Kss1pp/Hog1 Time=260 min**			**Kss1pp/Hog1 absolute increase in 260 min**			**Kss1pp/Hog1 fold increase in 260 min**		
Strain	**Mean**	**SE**	**p-value vs WT**	**Mean**	**SE**	**p-value vs WT**	**Mean increase**	**SE**	**p-value vs zero increase**	**fold increase**	**SE**	**p-value vs WT**
WT	0.233	3.3E-02	NA	0.993	0.033	NA	0.760	0.040	2.6E-24	4.297	0.205	NA
Δ*rgc1*	0.224	8.3E-02	8.7E-01	1.060	0.083	1.9E-01	0.836	0.056	9.6E-20	4.916	0.527	6.7E-02
Δ*ask10*	0.277	8.3E-02	3.8E-01	0.981	0.083	8.2E-01	0.704	0.056	7.8E-17	3.686	0.527	7.1E-02
Δ*rgc1*Δ*ask10*	0.232	8.3E-02	9.9E-01	1.099	0.083	3.9E-02	0.868	0.056	2.1E-20	4.959	0.527	5.2E-02
Δ*fps1*	0.231	8.3E-02	9.8E-01	1.116	0.083	1.8E-02	0.884	0.056	9.7E-21	4.967	0.527	4.9E-02
Δ*fus3*	0.626	8.3E-02	3.9E-10	3.091	0.083	7.0E-40	2.465	0.056	6.3E-41	5.016	0.527	3.6E-02
Δ*ssk1*	0.360	8.3E-02	1.5E-02	1.317	0.083	5.1E-08	0.957	0.056	3.7E-22	3.595	0.527	4.0E-02
Δ*sho1*	0.218	8.3E-02	7.7E-01	1.036	0.083	3.9E-01	0.818	0.056	2.3E-19	4.644	0.527	2.9E-01
Δ*ssk2*	0.352	8.3E-02	2.2E-02	1.356	0.083	3.3E-09	1.003	0.056	4.9E-23	3.763	0.527	1.1E-01
Δ*slt2*	0.313	8.3E-02	1.2E-01	1.271	0.083	1.3E-06	0.958	0.056	3.5E-22	3.948	0.527	2.9E-01
Δ*pea2*	0.255	8.3E-02	6.5E-01	0.921	0.083	1.6E-01	0.666	0.056	6.1E-16	3.516	0.527	2.4E-02
Δ*spa2*	0.265	8.3E-02	5.3E-01	0.860	0.083	1.1E-02	0.595	0.056	3.3E-14	3.165	0.527	1.9E-03
	Fus3pp/Hog1 Time=0			**Fus3pp/Hog1 Time=260 min**			**Fus3pp/Hog1 absolute increase in 260 min**			**Fus3pp/Hog1 fold increase in 260 min**		
Strain	**Mean**	**SE**	**p-value vs WT**	**Mean**	**SE**	**p-value vs WT**	**Mean increase**	**SE**	**p-value vs zero increase**	**fold increase**	**SE**	**p-value vs WT**
WT	0.393	3.1E-02	NA	4.744	0.347	NA	4.351	0.348	7.3E-17	12.634	1.202	NA
Δ*rgc1*	0.350	8.0E-02	3.9E-01	5.126	0.979	5.5E-01	4.775	0.530	5.7E-12	15.088	3.729	3.4E-01
Δ*ask10*	0.338	7.9E-02	2.6E-01	4.574	0.933	7.7E-01	4.235	0.475	7.6E-12	13.597	3.450	6.7E-01
Δ*rgc1*Δ*ask10*	0.352	8.0E-02	4.1E-01	5.333	0.996	3.7E-01	4.981	0.551	5.1E-12	15.078	3.727	3.4E-01
Δ*fps1*	0.290	7.5E-02	2.4E-02	4.794	0.951	9.3E-01	4.504	0.496	4.6E-12	16.610	4.039	1.7E-01
Δ*fus3*	0.110	6.3E-02	2.6E-11	0.141	0.694	7.8E-18	0.031	0.020	1.3E-01	1.274	2.405	3.4E-09
Δ*ssk1*	0.429	8.7E-02	5.2E-01	6.683	1.114	1.5E-02	6.255	0.686	4.0E-12	16.369	3.988	1.9E-01
Δ*sho1*	0.434	8.7E-02	4.7E-01	4.949	0.964	7.4E-01	4.515	0.513	1.2E-11	11.736	3.141	6.5E-01
Δ*ssk2*	0.455	8.9E-02	2.9E-01	7.376	1.177	2.6E-03	6.921	0.756	3.4E-12	16.515	4.019	1.8E-01

Δ*slt2*	0.743	1.2E-01	1.7E-04	8.167	1.249	4.1E-04	7.424	0.837	9.1E-12	11.390	3.088	5.2E-01
Δ*pea2*	0.571	1.0E-01	1.3E-02	5.013	0.969	6.7E-01	4.442	0.521	3.0E-11	8.829	2.759	2.3E-02
Δ*spa2*	0.408	8.5E-02	7.8E-01	5.449	1.006	2.9E-01	5.041	0.563	6.9E-12	13.452	3.424	7.2E-01

The mean and SE reported correspond to the fixed effects of the linear mixed-effects model used to analyze this data (see statistical methods for details).

Table S2. Yeast strains.

Strain	Relevant genotype	Reference
ACL3341	*MAT*a *bar1Δ prm1::P_{PRM1}-mCherry::hygB stl1::P_{STL1}-YFP::URA3*	This study
LD3342	*MAT*a *bar1Δ prm1::P_{PRM1}-mCherry::hygB stl1::P_{STL1}-YFP::URA3 bmh2::P_{BMH2}-CFP::TRP1*	This study
RB3376a	*MAT*a *mpk1Δ::kanMX, bar1Δ prm1::P_{PRM1}-mCherry::hygB stl1::P_{STL1}-YFP::URA3 bmh2::P_{BMH2}-CFP::TRP1*	This study
RB3704	*MAT*a *sho1Δ::kanMX, bar1Δ prm1::P_{PRM1}-mCherry::hygB stl1::P_{STL1}-YFP::URA3 bmh2::P_{BMH2}-CFP::TRP1*	This study
RB3382a	*MAT*a *ssk1Δ::kanMX, bar1 prm1::P_{PRM1}-mCherry::hygB stl1::P_{STL1}-YFP::URA3 bmh2::P_{BMH2}-CFP::TRP1*	This study
RB3642	*MAT*a *ssk2Δ::HisMX, bar1Δ prm1::P_{PRM1}-mCherry::hygB stl1::P_{STL1}-YFP::URA3 bmh2::P_{BMH2}-CFP::TRP1*	This study
RB3713	*MAT*a *fus3Δ::KanMX, bar1Δ prm1::P_{PRM1}-mCherry::hygB stl1::P_{STL1}-YFP::URA3 bmh2::P_{BMH2}-CFP::TRP1*	This study
RB3710	*MAT*a *rgc1Δ::KanMX, bar1Δ prm1::P_{PRM1}-mCherry::hygB stl1::P_{STL1}-YFP::URA3 bmh2::P_{BMH2}-CFP::TRP1*	This study
RB3717	*MAT*a *rgc2Δ::KanMX, bar1Δ prm1::P_{PRM1}-mCherry::hygB stl1::P_{STL1}-YFP::URA3 bmh2::P_{BMH2}-CFP::TRP1*	This study
RB3722	*MAT*a *rgc1Δ::KanMX rgc2Δ::HisMX bar1Δ prm1::P_{PRM1}-mCherry::hygB stl1::P_{STL1}-YFP::URA3 bmh2::P_{BMH2}-CFP::TRP1*	This study
RB3396a	*MAT*a *fps1Δ::His bar1Δ prm1::P_{PRM1}-mCherry::hygB stl1::P_{STL1}-YFP::URA3 bmh2::P_{BMH2}-CFP::TRP1*	This study
RB3406	*MAT*a *bar1Δ stl1::P_{STL1}-CFP::TRP1*	This study
RB3862	*MAT*a *spa2Δ::KanMX bar1Δ stl1::P_{STL1}-CFP::TRP1*	This study
RB3865	*MAT*a *pea2Δ::KanMX, bar1Δ prm1::P_{PRM1}-mCherry::hygB stl1::P_{STL1}-YFP::URA3 bmh2::P_{BMH2}-CFP::TRP1*	This study
RB3801	*MAT*a *hog1Δ::KanMX, bar1Δ prm1::P_{PRM1}-mCherry::hygB stl1::P_{STL1}-YFP::URA3 bmh2::P_{BMH2}-CFP::TRP1*	This study

ySP69	*MAT*a *HTA2::HTA2-CFP-no marker HOG1-Venus::TRP1 leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	(46)
yGP1281	*BY4742 s288c MAT*α *Pact1-RFP -URA3 in lyp1*	G. Pesce, Molecular Sciences Institute, CA